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Quantitative Analysis of the Degree of Silanization by the Ninhydrin Method and its Application to the Immobilization of GL-7-ACA Acylase and Cellulolytic Enzyme

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Abstract A simple quantitative method to measure the degree of silanization was developed, based on the reaction of ninhydrin with the silanization reagent (3-aminopropyltriethoxysilane, 3-APTES). At low concentrations (0.001–0.005%, v/v) of 3-APTES, a good linearity was obtained when 3-APTES reacted with undiluted ninhydrin for 30 min. On the other hand, at high levels of 3-APTES, a linearity was obtained when 3-APTES reacted with 3-fold diluted ninhydrin for 20 min. The reliability of regression curves mentioned above was expressed as a regression coefficient (R^2) of more than 0.99. Immobilization of different enzymes was introduced via silanization by using the 3-APTES in order to confirm the validity of the ninhydrin method. When yields for each step in the immobilization process were compared, yields of both glutaraldehyde and protein were found to have the same tendency to silanization. These results show that the ninhydrin method was suitable for quantitative analysis of silanization and that yields of immobilization could be pre-estimated by measuring silanization levels using the ninhydrin method.

Key words: Quantitative analysis, 3-APTES, ninhydrin, silanization

Enzyme immobilization on insoluble supports has been extensively developed in the industrial applications of enzymes [4-6]. One of the most important techniques for enzyme immobilization is the attachment of amino groups through modification of the carrier surface. The silanization is mostly used for modification of the carrier surface [3, 9]. For silanization, 3-aminopropyltriethoxylsilane (3-APTES) is a widely used reagent because its amino groups are susceptible to the coupling reaction [1, 10]. Although research activities dealing with silanization in various fields are extensive, a simple method that is generally accepted for direct quantitative analysis is lacking. In fact, many researchers have studied the methods used for quantitative analysis of the amino group. The ninhydrin method, in particular, is widely used for analyzing amino groups. Moore and Stein [8] developed a stable ninhydrin reagent, improved the reproducibility of the method, and established the reaction condition for amino acid analysis. Curotto and Aros [2] described the use of the ninhydrin reaction for quantitative determination of chitosan as well as the percentage of free amino groups. However, quantitative analysis of the degree of silanization by using the ninhydrin method has not yet been reported. The purpose of this study is to develop a method for measuring the degree of silanization through quantitative determination of 3-APTES. Also, in order to confirm the validity of the ninhydrin method, different enzymes such as GL-7-ACA acylase and cellulolytic enzymes were immobilized on the silica gel (XWP) and the binding yields of silanization, glutaraldehyde, and protein were compared.

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Materials and Methods

Materials

3-Aminopropyltriethoxysilane (3-APTES) and ninhydrin reagent were purchased from Sigma chemicals (St. Loius, U.S.A), glutaraldehyde from Fluka Co. (Buchs, Switzerland), and silica gel and GL-7-ACA acylase (glutaryl-7-aminocephalosporanic acid acylase) were supplied by Chong Kun Dang Pharmaceutical Corp. (Ansan, Korea). Novozyme 188 (β-glucosidase) and Cellusoft (endoglucanase) were purchased from Novo Co. (Denmark), and 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH)

was purchased from Aldrich Chemical Co. (Milwaukee, U.S.A).

Standard Solution of 3-APTES

Different concentrations of 3-APTES (0.005-0.05%, v/v) were used to examine the linearity.

Quantitative Analysis by the Ninhydrin Method

The ninhydrin method was based on the method described by Sabina *et al.* [13]. To 1 ml of 3-APTES solution in distilled water, 1 ml each of different concentrations of ninhydrin reagent were added and reacted in a covered boiling water bath at different times. The reactants were then cooled to below 30°C in a cold-water bath and the contents were then diluted with 5 ml of 50% (v/v) ethanol/water. The absorbance level at 570 nm was measured with a UV/VIS spectrophotometer (Youngwoo Corp., Seoul, Korea).

Pretreatment of Carriers

Carriers were pretreated with 35% hydrogen peroxide at 25°C for 2 h, and with a mixture of sulfuric acid and hydrogen peroxide at 80°C for 2 h, respectively. The ratio of sulfuric acid and hydrogen peroxide was 5:1 (v/v).

Immobilization of Enzyme

One gram of dry silica gels was mixed with 3-aminopropyltriethoxysilane and its pH was adjusted to 9.0 with 1 M HCl. The suspension was incubated at 75°C for 2 h with constant mixing, washed thoroughly with water before drying at 120°C for 2 h, and then 1% (v/v) glutaraldehyde was added to the suspended carriers in 100 mM of phosphate buffer (pH 8) at 20°C. After stirring (150 rpm) for 2 h, the suspension was filtered and carriers were washed with water. The carriers were resuspended in 100 mM of phosphate buffer (pH 8). Finally, an enzyme suspension was added.

Determination of the Amount of Glutaraldehyde Bound to the Carriers

The amount of glutaraldehyde bound to the carriers was determined by the difference between the amount of added glutaraldehyde and that measured in the solution, according to the Bersthorn's hydrozone method [12]. Two milliliters of an appropriately diluted sample were placed in a test tube and reacted at 100°C with 2 ml of 0.4% (w/v) MBTH. After 3 min, the mixture was rapidly cooled in an ice bath. Then, 5 ml of 0.4% (w/v) $FeCl_3$ was added. After 15 min at 25°C, an absorbance at 635 nm was measured with a UV/VIS spectrophotometer.

Determination of the Amount of Protein Bound to the Carriers

The amount of protein bound to the carriers was determined by the difference between initial and residual protein concentrations, using the Folin-Lowry method [7].

Calculation of Yields

Yields of each step in the immobilization process were calculated as the ratio of the amount bound on the silica gel to the initial amount, and were expressed as a percentage.

Results and Discussion

At Low Levels (0.001-0.005%, v/v) of 3-APTES

In order to determine the optimal reaction condition of 3-APTES with ninhydrin at low levels of 3-APTES, experiments were carried out at different reaction times. As shown in Fig. 1, when 3-APTES reacted with undiluted ninhydrin reagent for 30 min, a good linearity was observed. Reliability of the regression curves, expressed as the regression coefficient (R^2), was 0.995. However, at 10 and 20 min of the reaction time, the reliabilities of the regression curves were 0.968 and 0.982, respectively. Such a low reliability was believed to be caused by an insufficient reaction time. On the other hand, as shown in Fig. 2, when 3-fold diluted ninhydrin reagent was reacted with 3-APTES, the maximum

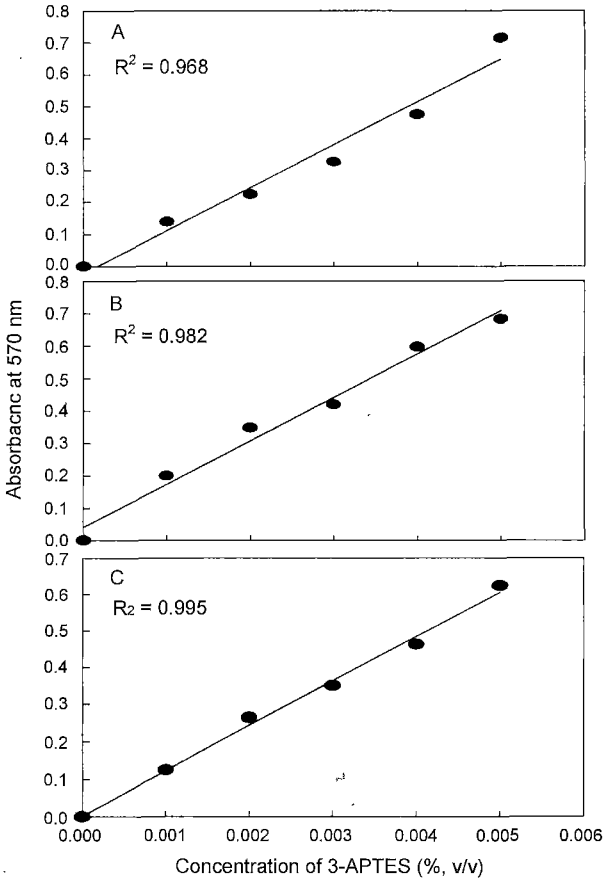

Fig. 1. Effect of reaction time on the quantitative analysis of 3-APTES at low levels of 3-APTES.
Experiments were carried out at 100°C with different reaction times (A-C) using undiluted ninhydrin reagent. A: 10 min; B: 20 min; C: 30 min.

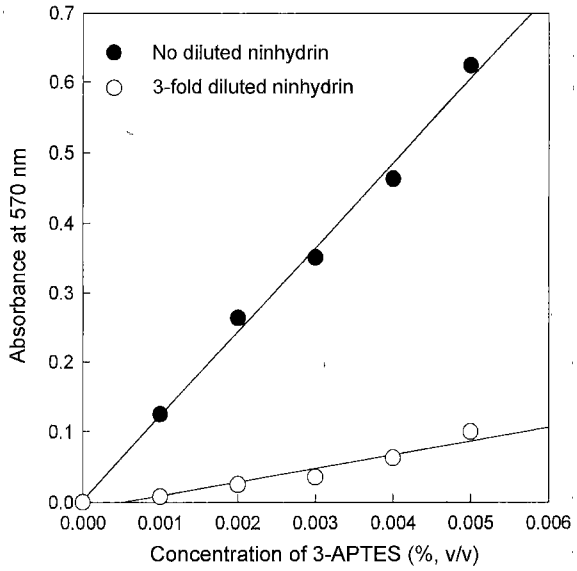

Fig. 2. Effect of ninhydrin concentrations at low levels of 3-APTES. Experiments were carried out, at 100°C, with different concentrations of ninhydrin reagent for 30 min.

value of absorbance was below 0.1. This value was indeed too low to be used in this study. Consequently, we concluded that the optimal reaction condition of 3-APTES with ninhydrin at a low level was to use undiluted ninhydrin reagent for 30 min.

At High Levels (0.01-0.05%, v/v) of 3-APTES

The optimal reaction condition at high levels of 3-APTES with ninhydrin was also investigated. When high concentrations of 3-APTES were reacted with undiluted ninhydrin reagent for 10 min, linearity was not observed at above 0.01% (v/v) (Fig. 3). However, when 3-APTES was reacted with 3-

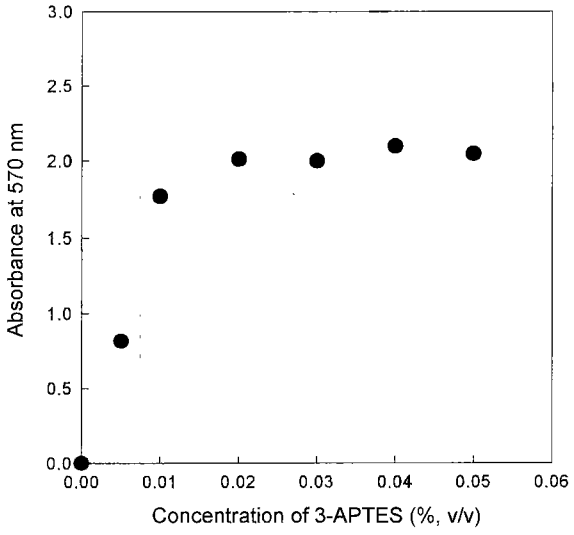

Fig. 3. Absorbance at high level concentrations of 3-APTES. 3-APTES was reacted with undiluted ninhydrin reagent for 10 min.

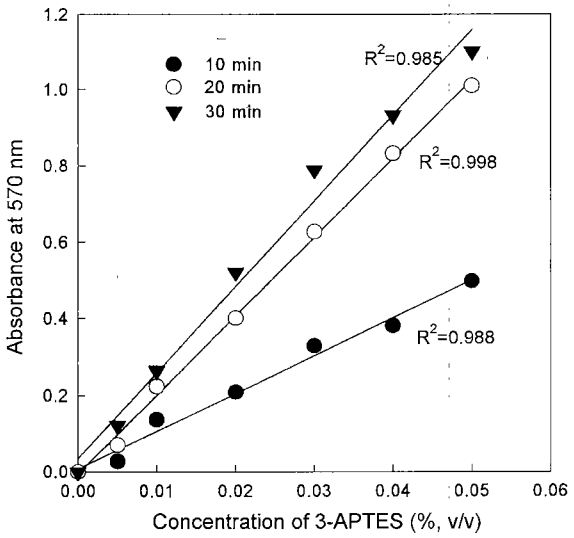

Fig. 4. Effect of reaction time at high levels of 3-APTES. Experiments were carried out for different reaction times using 3-fold diluted ninhydrin reagent.

fold diluted ninhydrin reagent for 20 min, good linearity was observed (Fig. 4). Reliability of the regression curves was 0.998. However, at 10 min of the reaction time, reliability of the regression curves was 0.988 and the absorbance range was below 0.6. These values were much lower than in the case of 20 min. At 30 min of reaction time, reliability of the regression curves was 0.985. This value was slightly lower than that in the case of 20 min. Such a value at 30 min might have been due to the excess reaction.

Overall, the reaction of 3-APTES with ninhydrin was quick, sensitive, and accurate. The linear calibration curves were obtained at both low and high levels of 3-APTES. In particular, at a high level of 3-APTES reacted with 3-fold diluted ninhydrin reagent for 20 min, the best linearity was observed. On the basis of these results, quantitative analysis to measure the degree of silanization was found to be possible by measuring of 3-APTES concentrations in the solution. That is, the amount of 3-APTES bound to the support was determined by measuring the difference between the amounts of added 3-APTES and that found in the solution by the ninhydrin method which was modified in our study. This method may also be suitable for determining the concentration of other amino group-containing reagents.

In order to confirm the validity of the ninhydrin method for the quantitative analysis of the silanization, immobilization of the enzyme was introduced via silanization by using the 3-APTES.

Application of the Ninhydrin Method in the Immobilization Process

To confirm the usefulness of the above results in a real life situation, GL-7-ACA acylase was immobilized on carriers which were pretreated with 35% of hydrogen peroxide and a mixture of sulfuric acid and hydrogen peroxide, respectively.

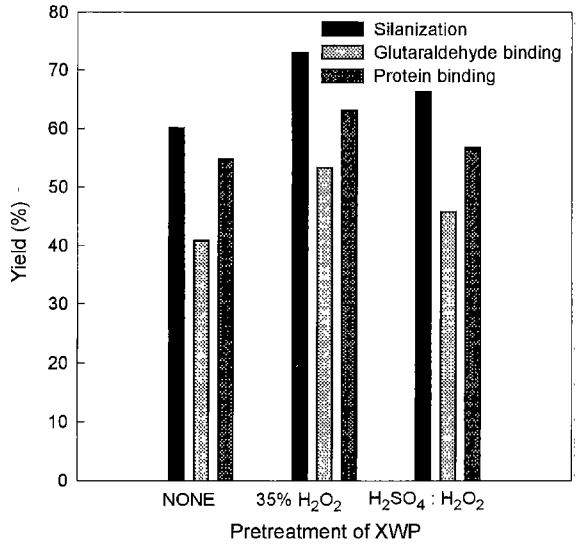

Fig. 5. Comparison of yields of each step on the immobilization of GL-7-ACA acylase.
Carriers were pretreated with different chemicals.

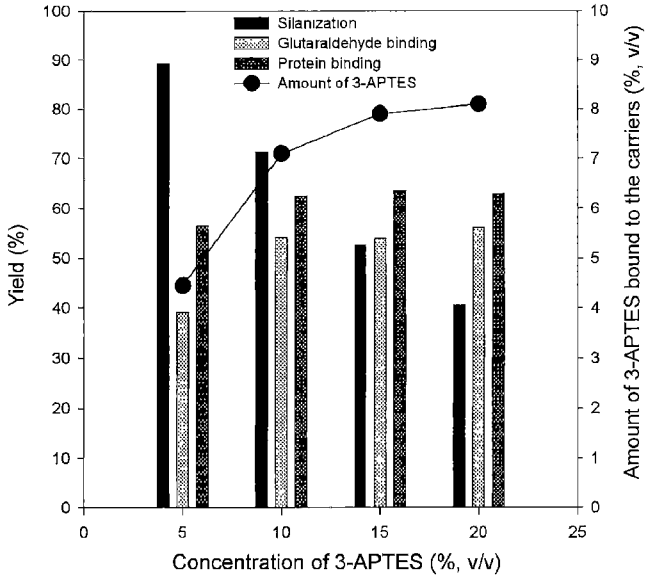

Fig. 6. Effect of 3-APTES concentrations on the immobilization process.
Silanization was carried out at different concentrations of 3-APTES.

Immobilization process was composed of three steps: silanization on the carriers, attachment of glutaraldehyde as the crosslinking agent [11], and binding of the enzyme. In this experiment, yields of each step in the immobilization process were compared to confirm the validity of the ninhydrin method. As shown in Fig. 5, the highest yields of silanization were obtained from XWP pretreated with 35% of hydrogen peroxide. Also, yields of both glutaraldehyde and protein bound on the carrier pretreated with 35% of hydrogen peroxide were higher than those made by others. These results showed the same tendency as that of the silanization yield, and indicated that the ninhydrin method was suitable for the quantitative analysis of silanization, and that yields of immobilization could be pre-estimated simply by measuring silanization levels using the ninhydrin method.

Effect of 3-APTES Concentrations on the Immobilization Process

In order to gain information on the effect of 3-APTES concentrations on the silanization, silanization was performed with different concentrations of 3-APTES, and the degree of silanization was then measured by the ninhydrin method. GL-7-ACA acylase was used in this experiment. As shown in Fig. 6, even though the yield of silanization was decreased with increasing concentrations of 3-APTES, the amount of 3-APTES bound on the carrier was increased up to 10% of 3-APTES. The amount of 3-APTES bound on the carrier was increased slightly at above a 10% concentration of 3-APTES. In comparison, the binding yields of both glutaraldehyde and protein showed quite similar behavior to that of 3-APTES bound on carrier. It is apparent from these results that the yields of each step in immobilization are dependent on the degree of silanization.

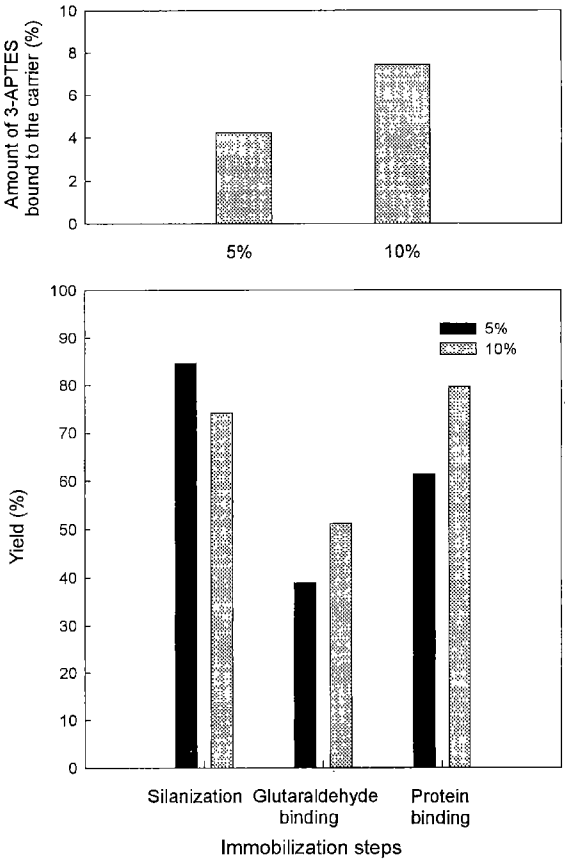

Fig. 7. Comparison of yields of each step on the immobilization of β-glucosidase.
Carriers were treated with different concentration (5% and 10%) of 3-APTES.

Application of Silanization in the Immobilization of Various Enzymes

In this study, for the purpose of wide application of silanization to immobilization of enzyme, β-glucosidase (Novozyme 188, Novo Co., Denmark) and endoglucanase (Cellusoft, Novo Co., Denmark) were immobilized on carriers which had been treated with different concentrations (5 and 10%, v/v) of 3-APTES. At this point, immobilization was carried out under an optimal condition that was achieved in our laboratory (data not shown). As shown in Figs. 7 and 8, when 5% and 10% (v/v) of 3-APTES were added, the amounts of 3-APTES bound on carriers were 4.23% (v/v) and 7.42% (v/v), respectively. Also, yields of silanization were calculated to be 84.6% and 74.2%, respectively. These values were similar to the above results (Fig. 6), thus confirming the validity of the ninhydrin method for the quantitative analysis of the silanization. When the carrier was treated with 10% of 3-APTES, glutaraldehyde and protein binding yields were higher than those made by the 5% concentration of 3-APTES. In particular, the highest yield of protein binding was obtained by endoglucanase.

Finally, the presently described results indicated that all experiments in this work demonstrated consistent tendency, implying that silanization could be applied in the immobilization of various enzymes. Particularly, the ninhydrin method to measure the degree of silanization could be used for predicting immobilization yields.

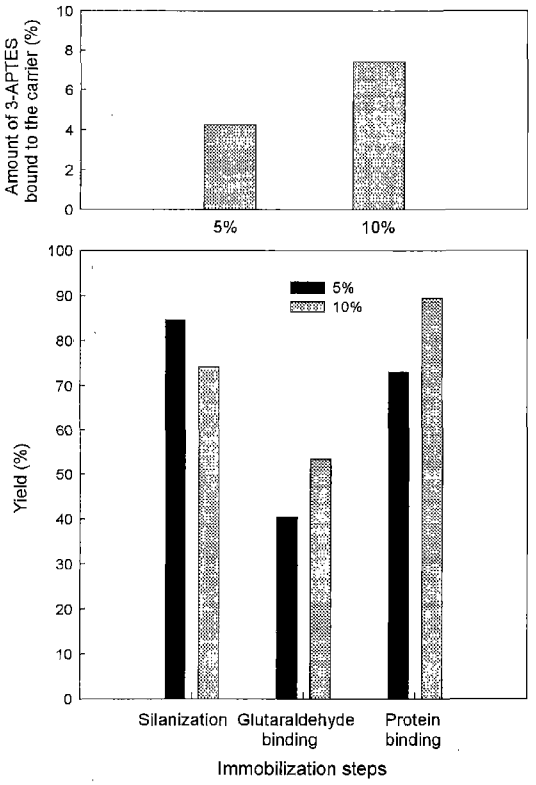

Fig. 8. Comparison of yields of each step on the immobilization of endoglucanase.
Carriers were treated with different concentration (5% and 10%) of 3-APTES.

Acknowledgment

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